

# Extrachromosomal Probes for Mutagenesis by Carcinogens: Studies on the Mutagenic Activity of O<sup>6</sup>-Methylguanine Built into a Unique Site in a Viral Genome

by John M. Essigmann,\* Kerry W. Fowler,\*† Calvert L. Green,‡ and Edward L. Loechler\*§

This work examines the mutagenic activity of O<sup>6</sup>-methylguanine (O<sup>6</sup>MeGua), a DNA adduct formed by certain carcinogenic alkylating agents. A tetranucleotide, 5'-HO-T<sub>p</sub>m<sup>6</sup>G<sub>p</sub>C<sub>p</sub>A-3', was synthesized and ligated into a four-base gap in the unique Pst I site of the duplex genome of the *E. coli* virus, M13mp8. The double-stranded ligation product was converted to single-stranded form and used to transform *E. coli* to produce progeny phage. The mutation frequency of O<sup>6</sup>MeGua was defined as the percentage of progeny phage with mutations in their Pst I site, and this value was determined to be 0.4%. To determine the impact of DNA repair on mutagenesis, cellular levels of O<sup>6</sup>MeGua-DNA methyltransferase (an O<sup>6</sup>MeGua-repair protein) were depleted by treatment of host cells for virus replication with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) prior to viral DNA uptake. In these host cells, the mutation frequency due to O<sup>6</sup>MeGua increased markedly with increasing MNNG dose (the highest mutation frequency observed was 20%). DNA sequence analysis of mutant genomes revealed that in both MNNG treated and untreated cells, O<sup>6</sup>MeGua induced exclusively G to A transitions.

## Introduction

Chemical and physical agents induce cancer by mechanisms that have only recently begun to be understood. The current view is that carcinogenic agents damage DNA, forming lesions that subsequently are acted upon by constitutive or inducible enzymatic systems to create mutations. These mutations could initiate carcinogenesis if they either cause, or create a favorable environment for the further genetic or nongenetic changes required for full development of the transformed phenotype.

Several independent lines of investigation have provided the rationale for suggesting a mutational origin for cancer. First, a strong empirical relationship exists between the mutagenic and carcinogenic effects of chemicals (1), with positive mutagenic activity being demonstrated for 80 to 90% of the carcinogens tested (2). Second, it has been observed that people with a genetic defect in one of the steps of DNA repair are at very high risk to certain forms of cancer (3); presumably

the inability of a cell to remove premutagenic lesions from DNA increases its likelihood of becoming the progenitor of tumor cell population. Finally, much has been discovered recently as to the identities of some of the genes apparently involved in the origins of some human and animal cancers (4,5). These studies have shown that some oncogenes appear to be variants of normal cellular genes that are likely to have undergone mutation. Taken together, all of the evidence cited above presents a good case in support of somatic mutations being responsible for at least some cancers.

In view of the relatively firm relationship that exists between DNA modification and mutagenesis, and the further, albeit more tentative association between mutagenesis and carcinogenesis, it has become of interest to elucidate the specific mechanisms by which mutations are induced by the DNA lesions caused by chemical carcinogens. One factor that greatly complicates this analysis derives from the observation that the DNA-damaging forms of carcinogens generally create a wide variety of structurally different lesions within the genome (6-10). Multiple DNA-bound forms of carcinogens, herein referred to as adducts, result in part because the pathways of carcinogen activation often generate several chemically distinct reactive species (6). In addition, the presumed relevant target for these species, DNA,

\*Laboratory of Toxicology, Department of Nutrition and Food Science and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.

†Present address: G. D. Searle and Co., Chicago, IL 60680.

‡Present address: Biology Department, Boston, MA 02215.

§Present address: AMGen, Inc., Thousand Oaks, CA 91320-1789.

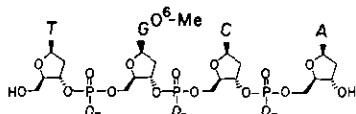


FIGURE 1. Structure of the chemically synthesized tetranucleotide, 5'-d( $T_p m^6 G_p C_p A$ )-3'.

is not a simple reactant, but a composite made up of at least 20 reactive centers that differ not only as to their inherent chemical reactivity but also as to their steric accessibility to activated carcinogens. Understandably, the result of carcinogen-DNA interactions usually is a structurally heterogeneous array of DNA adducts. Moreover, as a further complicating factor, this profile often varies markedly over time because some DNA adducts are removed rapidly whereas others are removed slowly if at all.

The multiplicity of DNA adducts created by the binding of carcinogens to DNA and the fact that the adduct profile changes over time make it difficult to define the relationship between the chemical structure of an individual adduct and its biological effects. The approach our laboratory has taken for establishing such relationships is to situate individual adducts at defined sites in genomes, allow enzymatic processing to occur *in vivo*, and then assess the genetic effects of the adduct by measuring the amount and type of DNA sequence alteration(s) induced at or in the vicinity of the site originally occupied by the adduct.

The prototype we chose for these studies is  $O^6$ -methylguanine ( $O^6$ MeGua), which is one of the DNA adducts formed by certain carcinogenic alkylating agents. This lesion was chosen for several reasons. First, the presence and persistence of the  $O^6$ -alkylguanines correlate well with the mutagenic and carcinogenic effects of alkylating agents (11-13). Second, the reaction of alkylating agents with the  $O^6$ -atom of guanine fixes the enol tautomer of this base, in which guanine has been predicted to base-pair with thymine as well as cytosine (14). Much data from *in vitro* systems support this prediction (15-18). Third, as demonstrated below, the chemical properties of this base made it an acceptable substrate for chemical synthesis of DNA. Finally, it was anticipated that this adduct would cause only minor distortion of DNA structure, and thus it could be built into DNA in high yield by using recombinant DNA techniques.

## Chemical Synthesis of an Oligonucleotide Containing $O^6$ -Methylguanine

For reasons made clear in the next section, it was our objective to build  $O^6$ MeGua into DNA as part of the tetranucleotide, 5'-d( $T_p m^6 G_p C_p A$ )-3' (Fig. 1) (19). Initially,  $O^6$ -methyldeoxyguanosine was prepared by the condensation of 2-amino-6-chloropurine with 2-deoxy-3,5,*O*-*p*-toluoyl-D-erythropentose chloride, followed by

*N*-acylation, fractional crystallization of the  $\beta$ -anomer, and methanolysis. Subsequent reactions yielded the  $O^6$ -methyldeoxyguanosine residue protected at the 5'-hydroxyl by a dimethoxytrityl group and at the 3'-hydroxyl by a *p*-chlorophenylcyanoethyl phosphotriester moiety. The strategy for assimilating this 3',5'-protected adduct into an oligonucleotide was initially to prepare two dimers, fully protected derivatives of 5'- $T_p m^6 G$ -3' and 5'- $C_p A$ -3'. The 3' and 5' ends of the first and second dimers, respectively, were deprotected and condensed using mesitylenesulfonyl tetrazolide, yielding a protected tetranucleotide. Removal of the protecting groups produced the adduct-containing tetranucleotide, 5'- $T_p m^6 G_p C_p A$ -3' (Fig. 1), which was structurally identified by its mass and NMR spectra, and by chemical composition and sequence studies.

## Site-Specific Incorporation of $O^6$ -Methylguanine into a Viral Genome

The DNA vector chosen for the biological aspect of these studies was the *E. coli* virus, M13mp8. This virus was chosen because its DNA sequence is known (20) and the biology of its replication is well understood (21), because it is relatively easy to produce the DNA needed for manipulation using recombinant DNA techniques, and because its DNA replication mechanism provides the option of constructing either single-stranded (ss) or double-stranded (ds) adduct-containing genomes.

In order to build the adduct-containing tetranucleotide into M13mp8, initially it was our objective to remove a 5'- $T_p G_p C_p A$ -3' segment from the circular duplex genome; these four bases are located within the unique recognition site for the restriction endonuclease, Pst I. The first step in this procedure (22) was to treat the replicative form (RF) of M13mp8 with Pst I (Fig. 2). This produced a linear DNA molecule with 3'-overhanging ends, which subsequently were removed by the 3'-exonuclease activity of T4 DNA polymerase. The product was a blunt-ended linear genome 7225 base pairs (bp) in length, i.e., the length of the genome minus four bp. The missing four nucleotides were originally present in the center of the Pst I recognition sequence (5'CTGCAG3').

A circular DNA structure containing a four-nucleotide gap was created by forming a heteroduplex between the aforementioned DNA molecule (from which the TGCA sequence had been deleted) and a second linear DNA molecule that had an intact Pst I site. The heteroduplex was formed by combining the linear product of the blunt-ending reaction with M13mp8 DNA that previously had been linearized with Bgl II; heat denaturation followed by controlled cooling of the mixture resulted in approximately equal amounts of the original linear structures (not shown as products in Fig. 2A) and circular heteroduplexes. In these heteroduplexes there was an exactly positioned four-nucleotide gap in the strand originally treated with Pst I and then blunt-ended. The intact DNA strand opposite the gap had the

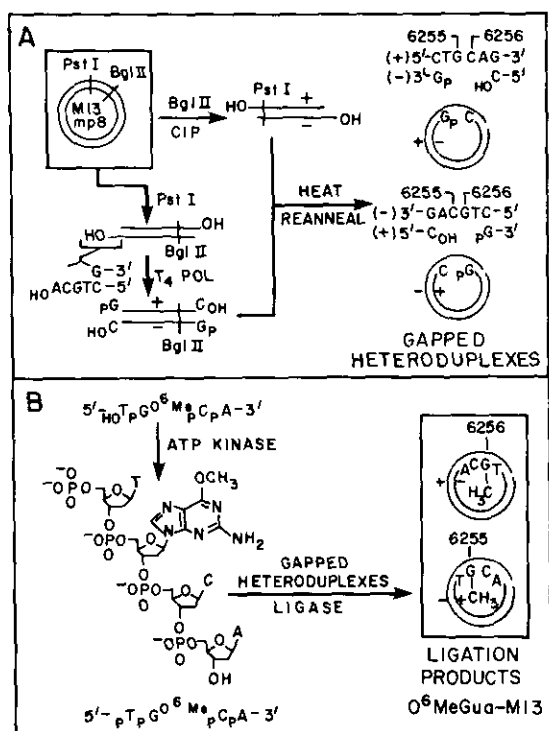


FIGURE 2. Site-specific insertion of O<sup>6</sup>MeGua into the unique Pst I recognition site of M13mp8. Procedural details are given in the text. CIP is calf intestinal alkaline phosphatase.

sequence 3'-A<sub>p</sub>C<sub>p</sub>G<sub>p</sub>T<sub>p</sub>-5' and thus displayed partial complementarity to the chemically synthesized 5'-T<sub>p</sub>m<sup>6</sup>G<sub>p</sub>C<sub>p</sub>A-3' molecule described above.

The 5'-terminal deoxythymidine of the synthetic tetranucleotide was phosphorylated by using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (Fig. 2B) prior to the ligation step. As judged by HPLC analysis of the reaction mixture, 97% phosphorylation was achieved. The high specific activity of 5'-[<sup>32</sup>P]-T<sub>p</sub>m<sup>6</sup>G<sub>p</sub>C<sub>p</sub>A-3' (approximately 10<sup>9</sup> dpm/ $\mu$ g) provided a means for measuring the yield of the ligation step. In the presence of T4 DNA ligase and a 2000-fold molar excess of 5'-pTpGpCpA-3', the yield of ligation into gapped duplex DNA was approximately 50%. This high yield indicated that the tetramer, when present in large excess, was sufficiently complementary to the ligation target for DNA ligase to very effectively form phosphodiester bonds. Electrophoresis followed by autoradiography of the ligation mixture revealed that incorporation of [<sup>32</sup>P] occurred principally in the circular heteroduplexes (22).

The heteroduplex DNA structures that received the modified oligonucleotide had gaps that formed with equal probability in either of the two complementary strands (Fig. 2A). Thus, ligation occurred into these gaps in either of two possible orientations, resulting in placement of O<sup>6</sup>MeGua at position 6255 when it was ligated into the (+) strand and at position 6256 when it was introduced into the (-) strand (Fig. 2B). It is noteworthy that the DNA strand opposite the gaps had a nick in

the Bgl II recognition site. This nick resulted from an optional step in which M13mp8 linearized with Bgl II was treated with calf intestinal alkaline phosphatase (CIP) prior to heteroduplex formation. This treatment removed the 5'-terminal phosphate residues and made it impossible for DNA ligase to seal the nick at the Bgl II site in the circular heteroduplex shown in Figure 2A. The existence of this nick provided a means for removing the strand complementary to the one containing the adduct, thereby creating single-stranded (ss) adduct-containing genomes.

The adduct-containing genomes shown as ligation products at the bottom of Figure 2B were subjected to a series of characterization experiments (22). One of these experiments established that both ends of the modified tetranucleotide had ligated into the gap originally present in the duplex genome; it was important that this step went to completion since failure of one of the ends to ligate would have left a nick in the DNA molecule near the adduct, and such a nick could have provided a mechanism for the efficient repair of the adducted genome upon its entry into cells. The position of the adduct was localized to the Pst I site of M13mp8 by the following experiments. After digestion of the modified genome with a restriction endonuclease with multiple recognition sites in M13mp8, it was found that the <sup>32</sup>P-label from the 5'-end of the tetranucleotide was present exclusively within the restriction fragment that contained the Pst I site. It was also demonstrated that the presence of O<sup>6</sup>MeGua in the Pst I site inhibited the ability of the Pst I endonuclease to cleave DNA. However, sensitivity to Pst I cleavage could be restored if the DNA was treated, prior to Pst I treatment, with a purified repair protein that specifically removed the O<sup>6</sup>-methyl group from guanine. Collectively, the data on characterization of the adduct-containing genome indicated that O<sup>6</sup>-MeGua was located with good yield and purity at the predicted site in the M13mp8 genome.

## Mutation Frequency of O<sup>6</sup>-Methylguanine *in Vivo*

As indicated above, ds genomes were constructed that contained a single O<sup>6</sup>MeGua residue within the unique Pst I site of M13mp8. There was a nick situated in the strand opposite that containing the adduct (Fig. 2B), and thus alkali denaturation yielded single-stranded monoadducted genomes (Fig. 3). It was desirable to have the option to remove the complementary strand, because it has been reported that the major repair protein for O<sup>6</sup>MeGua is less active on ss DNA than on double-stranded (ds) substrates (23).

To investigate mutagenesis by O<sup>6</sup>MeGua, ss O<sup>6</sup>MeGua-M13mp8 was introduced into *E. coli* MM294A cells, where the phage genomes were acted upon by the endogenous replication and repair systems (Fig. 3) (24). A mixture of wild type and mutant phage was produced, which was used to produce the ds replicative form (RF) DNA. The method used to differentiate mutant and wild

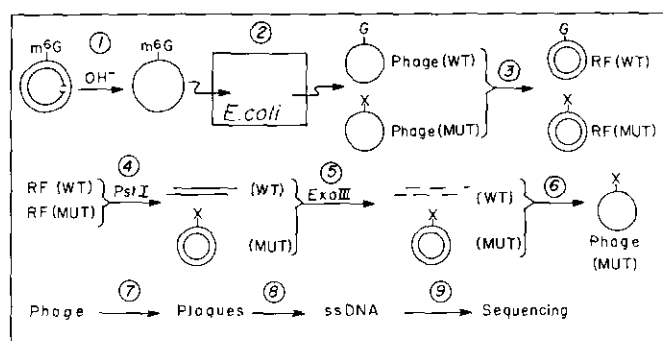


FIGURE 3. Isolation of  $O^6$ MeGua-derived mutants. ds  $O^6$ MeGua-M13mp8 was base-denatured to give ss  $O^6$ MeGua-M13mp8 (step 1), which then was used to transform *E. coli* MM294A cells (step 2). A mixture of wild type and mutant phage was produced (X denotes position of mutation) and used to infect *E. coli* JM103 cells. RF DNA prepared from these phage (step 3) was treated with Pst I (step 4); mutant DNA remained circular, whereas wild type molecules were linearized and subsequently selectively degraded with exonuclease III (step 5). Steps 4 and 5 were repeated, and the remaining DNA (an essentially pure mutant population) was retransfected into JM103 cells to produce phage (step 6). The ss phage genome was isolated for DNA sequencing (steps 7–9).

type phage was based on the fact that the progenitor phage DNA molecule (step 1 of Fig. 3) contained the adduct in the unique Pst I recognition site. Mutations affecting this site rendered the RF DNA insensitive to cleavage by this endonuclease. This property made it possible to isolate a pure mutant phage population for DNA sequencing and for calculation of mutation frequencies.

The mutation frequency of  $O^6$ MeGua was expressed as the fraction of phage produced after step 2 of Figure 3 with mutations in the Pst I site (24). As indicated by the DNA sequencing results (see below), the mutations induced by  $O^6$ MeGua were at either of two guanines in this site, because as indicated in the previous section  $O^6$ MeGua-M13mp8 was an equal mixture of genomes with the single adduct located either in the (+) strand (position 6255) or in the (–) strand (position 6256) (Fig.

Table 1. Mutation frequency (%) due to  $O^6$ MeGua in  $O^6$ MeGua-M13mp8.

MNNG challenge, $\mu\text{g/mL}^a$	$O^6$ MeGua-M13mp8			M13mp8, MF <sup>c</sup>
	MF <sup>–b</sup>	MF <sup>++b</sup>	MF <sup>tb</sup>	
0	0.08	0.36	0.4	$\leq 0.03$
17	1.3	4.1	5.4	$\leq 0.11$
33	3.6	4.7	8.3	$\leq 0.20$
50	4.1	13.7	17.8	$\leq 0.16$

<sup>a</sup> Some host cells for  $O^6$ MeGua-M13mp8 replication were challenged with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) prior to DNA uptake; details are given in the text.

<sup>b</sup> MF<sup>–</sup> and MF<sup>++</sup> are the percentage of progeny phage with  $O^6$ MeGua-derived mutations that originated in the (+) and (–) strands, respectively; the sum of these quantities is MF<sup>t</sup>.

<sup>c</sup> The upper limit of the mutation frequency of a control, which was the (+) strand of M13mp8 (this DNA did not contain  $O^6$ MeGua at the Pst I site).

2B). As shown in Table 1, the mutation frequencies of the adduct at these sites (MF<sup>–</sup> and MF<sup>++</sup>, respectively) were determined to be 0.36 and 0.08%, respectively. In calculating these values it was assumed that an adduct at a given site gave rise to a mutation at that site; i.e., ss  $O^6$ MeGua-M13mp8 with the adduct in the (+) strand gave rise to the mutation observed at position 6255. The sum of the values for the mutation frequencies in the individual strands is defined as the total mutation frequency of  $O^6$ MeGua, MF<sup>t</sup>, which was determined to be approximately 0.4%.

## Role of DNA Repair in Protecting against $O^6$ -Methylguanine Mutagenesis

Others have investigated the miscoding characteristics of  $O^6$ MeGua *in vitro* in experiments that typically have involved random incorporation of  $O^6$ -substituted guanines into DNA or RNA polymers, which then were copied with polymerases (15–17). Subsequently, the replication products were analyzed for the presence of noncomplementary nucleotides. The results of these experiments have demonstrated that DNA and RNA polymerases misreplicate  $O^6$ -alkylguanines approximately one-third of the time. Although less work has been done to estimate the mutation frequency *in vivo*, the few data that are available indicate a similar (25) or slightly higher (26) mutation frequency as compared to that observed *in vitro*. There must, however, be an element of uncertainty as to the accuracy of these *in vivo* estimates, because the experiments were done with DNAs containing the full range of adducts created by treatment with alkylating agents.

The mutation frequency we observed *in vivo* is several orders of magnitude less than that determined or predicted by the experiments cited above. The most likely reason for this apparent discrepancy is the fact that in our studies a single adduct was built into the genome, and this single lesion probably was removed quickly in *E. coli* by repair proteins associated with the adaptive response, specifically, the  $O^6$ MeGua DNA-methyltransferase (MT). This protein acts by transferring the methyl group from  $O^6$ MeGua in DNA to itself (27); the alkylated protein is not thought to turn over, and thus it is irreversibly inactivated in the process of dealkylating the genome.

We took advantage of the suicidal property of the MT to diminish the intracellular capacity to repair  $O^6$ MeGua. At 2 min before the  $O^6$ MeGua-M13mp8 uptake step (step 2 of Fig. 3), host cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which introduced  $O^6$ MeGua residues into the host chromosome (13). Repair of these lesions depleted endogenous levels of the MT and thus diminished the ability of cells to repair the single adduct in  $O^6$ MeGua-M13mp8. MNNG is known to induce several DNA repair systems, but we estimate that fixation of  $O^6$ MeGua as a mutation would occur

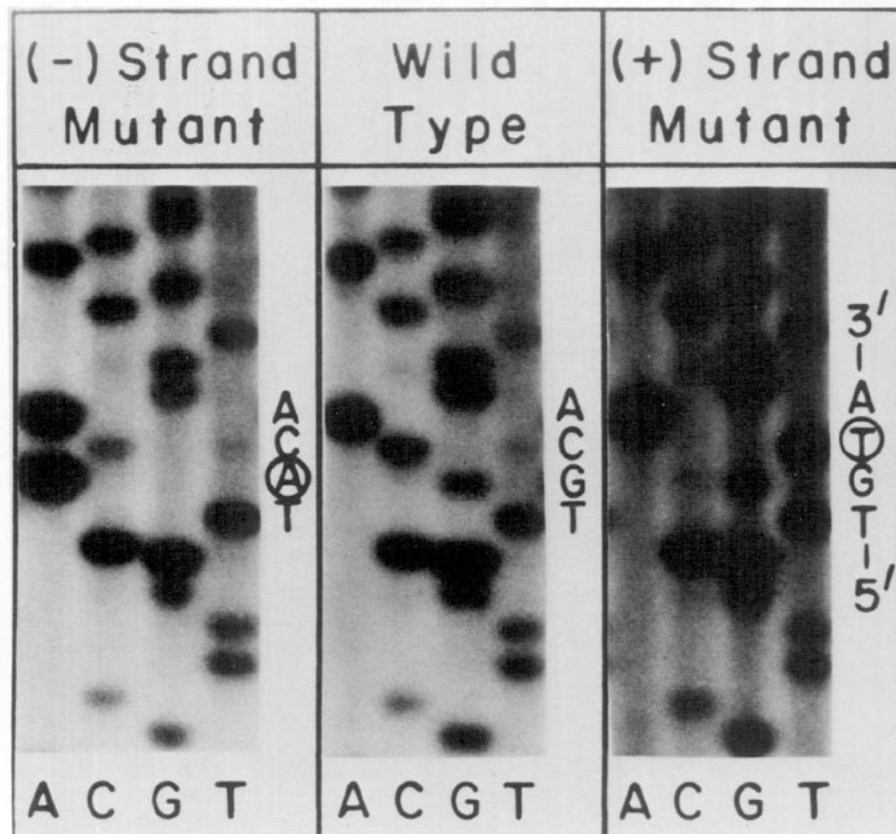


FIGURE 4. DNA sequences of O<sup>6</sup>MeGua-induced mutants. DNA of mutant phage was isolated and sequenced by the method of Sanger. Shown here are autoradiograms of sequencing gels in the region of the M13mp8 genome containing the Pst I sites of (from left to right) (-) strand mutant, wild type, and (+) strand mutant genomes.

before their induction, and thus the effect of these inducible systems on mutagenesis should be minimal. (We assume that O<sup>6</sup>MeGua-M13mp8 is replicated at the same rate as similar ss viruses, and thus it is estimated that the replication apparatus will encounter the adducted site within 2 min).

Table 1 presents the results of an experiment in which the mutation frequency of O<sup>6</sup>MeGua was examined in a series of cell populations that had been pretreated with a range of increasing doses of MNNG, i.e., treatments that created a range of reduced MT activities within the host cells. As expected the mutant fraction derived from O<sup>6</sup>MeGua-M13mp8 increased with the level of MNNG treatment. As a control to the above experiment, *E. coli* cells challenged identically with MNNG were transformed with wild type M13mp8 DNA. Mutation of this DNA to Pst I insensitivity was insignificant (Table 1), and this ensured that the observed mutations did not arise from alkylation of the vector during MNNG challenge.

At the highest level of MNNG treatment (50 µg/mL), the total MF (MF') had increased to almost 50 times the comparable value in unchallenged cells. The mutation frequency of this sample (approximately 20%) does not

necessarily represent the inherent mutation efficiency of O<sup>6</sup>MeGua, because the function relating MNNG dose and mutagenesis (Table 1) was still ascending at this level of MNNG challenge; rather, this value represents the lower limit of the true mutation frequency of this lesion. Interestingly, at its present value this mutation frequency is at the lower end of the range measured for mutagenesis of O<sup>6</sup>-alkyl guanines *in vitro* (15-17), and it is within a factor of two or three of the level predicted by indirect *in vivo* measurements (25,26).

### Nature of Mutations Induced by O<sup>6</sup>-Methylguanine

After the mutant isolation procedure was completed (i.e., after step 6 of Fig. 3) and individual mutant plaques were obtained, these mutant species were characterized by DNA sequencing. In addition to the two O<sup>6</sup>MeGua-induced mutant species indicated below, DNA sequencing revealed two other mutational changes. Specifically, two types of deletion mutant were induced (four-base, and approximately 30 base deletions); these appear to have originated from the genetic engineering tech-

niques used in the construction of the adducted genome, and not from mutagenic processing of O<sup>6</sup>MeGua (24). These mutant species were readily distinguishable from O<sup>6</sup>MeGua-induced mutants.

Figure 4 contains an autoradiogram of a DNA sequencing gel and reveals the DNA sequences of the wild type and of the two O<sup>6</sup>MeGua-induced mutant species in the vicinity of the Pst I site of M13mp8. The only sequence change in the left-most lanes was a G to A transition at position 6256. We assume this was due to misreplication of the ss O<sup>6</sup>MeGua-M13mp8 genome in which the adduct was located in the (-) strand (see Fig. 2B). Presumably, O<sup>6</sup>MeGua base-paired with thymine during synthesis of (+) strand *in vivo*, creating a phage population with T rather than C opposite the original position of the lesion. The rightmost lanes show the sequence of the mutant we assume issued from the O<sup>6</sup>MeGua-M13mp8 genome in which the adduct was in the (+) strand. The sequencing data indicate that a C to T change had occurred at position 6255; since the sequence of the (-) strand appears in the autoradiogram, this means that in the complementary (+) strand a G to A change had occurred. Thus the sequencing data are consistent with the original prediction based on model building (14) and *in vitro* data (cited above) that O<sup>6</sup>MeGua would induce G to A transitions. A total of 60 mutants have been sequenced, and all have shown this base change.

This work was supported by NIH Grants 5 P01 ES00597, T 32 ES07020, CA 33821, GM 09869 (to Professor G. H. Büchi), and by the Monsanto Fund.

## REFERENCES

- Meselson, M., and Russell, K. Cold Spring Harbor Conf. Cell Proliferation 4: 1473-1481 (1977).
- McCann, J. and Ames, B. N. Detection of carcinogens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. (U.S.) 73: 950-954 (1976).
- Cleaver, J. E., and Bootsma, D. *Xeroderma pigmentosum*: biochemical and genetic characteristics. Ann. Rev. Genet. 9: 19 (1975).
- Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R., and Chang, E. H. Mechanism of activation of a human oncogene. Nature 300: 143-149 (1982).
- Sukumar, S., Notario, V., Martin-Zanca, D., and Barbacid, M. Induction of mammary carcinomas in rats by nitroso-methylurea involves malignant activation of H-ras-1 locus by single point mutations. Nature 306: 658-661 (1983).
- Miller, E. C. Some current perspectives on chemical carcinogenesis in humans and experimental animals: presidential address. Cancer Res. 38: 1479-1496 (1978).
- Singer, B., and Kusmierek, J. T. Chemical mutagenesis. Ann. Rev. Biochem. 52: 655-693 (1982).
- Lawley, P. D. Approaches to chemical dosimetry in mutagenesis and carcinogenesis: the relevance of reactions of chemical mutagens and carcinogens with DNA. In: Chemical Carcinogens and DNA, Vol. 1 (P. L. Grover, Ed.), CRC Press, Boca Raton, FL, 1979, pp. 1-36.
- Essigmann, J. M., Green, C. L., Croy, R. G., Fowler, K. W., Büchi, G. H., and Wogan, G. N. Interactions of aflatoxin B<sub>1</sub> and alkylating agents with DNA: structural and functional studies. Cold Spring Harbor Symp. Quant. Biol. 47: 327-337 (1983).
- Conney, A. H. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. Cancer Res. 42: 4875-4917 (1982).
- Goth, R., and Rajewsky, M. F. Persistence of O<sup>6</sup>-ethylguanine in rat-brain DNA: correlation with nervous system-specific carcinogenesis by ethylnitrosourea. Proc. Natl. Acad. Sci. (U.S.) 71: 639-643 (1974).
- Kleihues, P., and Margison, G. P. Carcinogenicity of N-methyl-N-nitrosourea: possible role of excision repair of O<sup>6</sup>-methylguanine from DNA. J. Natl. Cancer Inst. 53: 1839-1841 (1974).
- Schendel, P. F., and Robins, P. E. Repair of O<sup>6</sup>-methylguanine in adapted *Escherichia coli*. Proc. Natl. Acad. Sci. (U.S.) 75: 6017-6020 (1978).
- Loveless, A. Possible relevance of O<sup>6</sup>-alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. Nature 223: 206-207 (1969).
- Gerchman, L. L., and Ludlum, D. B. The properties of O<sup>6</sup>-methylguanine in templates for RNA polymerase. Biochim. Biophys. Acta 308: 310-316 (1973).
- Mehta, J. R., and Ludlum, D. B. Synthesis and properties of O<sup>6</sup>-methyldeoxyguanylic acid and its copolymers with deoxycytidylic acid. Biochim. Biophys. Acta 512: 770-778 (1978).
- Abbott, P. J., and Saffhill, R. DNA synthesis with methylated poly(dC-dG) templates. Evidence for a competitive nature to mis-coding by O<sup>6</sup>-methylguanine. Biochim. Biophys. Acta 562: 51-61 (1979).
- Dodson, L. A., Foote, R. S., Mitra, S., and Masker, W. E. Mutagenesis of bacteriophage T7 *in vitro* by incorporation of O<sup>6</sup>-methylguanine during DNA synthesis. Proc. Natl. Acad. Sci. (U.S.) 79: 7440-7444 (1982).
- Fowler, K. W., Büchi, G., and Essigmann, J. M. Synthesis and characterization of an oligonucleotide containing a carcinogen-modified base: O<sup>6</sup>-methylguanine. J. Am. Chem. Soc. 104: 1050-1054 (1982).
- Van Wezenbeck, P. M. G. F., Hulsebos, T. J. M., and Schoenmakers, J. G. G. Nucleotide sequence of the filamentous bacteriophage M13 DNA genome: comparison with phage fd. Gene 11: 129-148 (1980).
- Messing, J. New M13 vectors for cloning. Methods Enzymol. 101: 20-78 (1983).
- Green, C. L., Loechler, E. L., Fowler, K. W., and Essigmann, J. M. Construction and characterization of extrachromosomal probes for mutagenesis by carcinogens: site-specific incorporation of O<sup>6</sup>-methylguanine into viral and plasmid genomes. Proc. Natl. Acad. Sci. (U.S.) 81: 13-17 (1984).
- Lindahl, T., Demple, B., and Robins, P. Suicide inactivation of the *E. coli* O<sup>6</sup>-methylguanine-DNA methyltransferase. EMBO J. 1: 1359-1363 (1982).
- Loechler, E. L., Green, C. L., and Essigmann, J. M. *In vivo* mutagenesis by O<sup>6</sup>-methylguanine built into a unique site in a viral genome. Proc. Natl. Acad. Sci. (U.S.) 81: 6271-6275 (1984).
- Lawley, P., and Martin, C. Molecular mechanisms in alkylation mutagenesis. Biochem. J. 145: 85-91 (1975).
- Guttenplan, J. Mutagenesis and O<sup>6</sup>-ethylguanine levels in DNA from N-nitroso-N-ethylurea-treated *Salmonella typhimurium*: evidence for a high mutational efficiency of O<sup>6</sup>-ethylguanine. Carcinogenesis 5: 155-159 (1984).
- Olsson, M., and Lindahl, T. Repair of alkylated DNA in *Escherichia coli*. J. Biol. Chem. 255: 10569-10571 (1980).